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Inhibition of the kynurenine pathway protects against reactive microglial-associated reductions in the complexity of primary cortical neurons

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ABSTRACT

Brain glia possess the rate limiting enzyme indoleamine 2, 3-dioxygenase (IDO) which catalyses the conversion of tryptophan to kynurenine. Microglia also express kynurenine monooxygenase (KMO) and kynureninase (KYNU) which lead to the production of the free radical producing metabolites, 3-hydroxykynurenine and 3-hydroxyanthranillic acid respectively and subsequently production of the NMDA receptor agonist quinolinic acid. The aim of this study was to examine the effect of IFNγ-stimulated kynurenine pathway (KP) induction in microglia on neurite outgrowth and complexity, and to determine whether alterations could be abrogated using pharmacological inhibitors of the KP. BV-2 microglia were treated with IFNγ (5 ng/ml) for 24 h and conditioned media (CM) was placed on primary cortical neurons 3 days *in vitro* (DIV) for 48 h. Neurons were fixed and neurite outgrowth and complexity was assessed using fluorescent immunocytochemistry followed by Sholl analysis. Results show increased mRNA expression of IDO, KMO and KYNU, and increased concentrations of tryptophan, kynurenine, and 3-hydroxykynurenine in the CM of IFNγ-stimulated BV-2 microglia. The IFNγ-stimulated BV-2 microglial CM reduced neurite outgrowth and complexity with reductions in various parameters of neurite outgrowth prevented when BV-2 microglia were pre-treated with either the IDO inhibitor, 1-methyltryptophan (1-MT) (L) (0.5 mM; 30 min), the KMO inhibitor, Ro 61–8048 (1 μM; 30 min), the synthetic glucocorticoid, dexamethasone (1 μM; 2 h) -which suppresses IFNγ-induced IDO - and the N-methyl-D-aspartate (NMDA) receptor antagonist, MK801 (0.1 μM; 30 min). Overall this study indicates that inhibition of the KP in microglia may be targeted to protect against reactive microglial-associated neuronal atrophy.

1. Introduction

The kynurenine pathway (KP) is a prominent route for tryptophan metabolism that yields multiple metabolites with pharmacologically active properties on neurons (Schwarcz et al., 2012). This includes metabolites that exert an influence at both N-methyl-D-aspartate (NMDA) and metabotropic glutamate receptors and also the aryl hydrocarbon receptor and specific G protein coupled receptors (Cuartero et al., 2014; Wang et al., 2006). There is growing recognition for a role for the KP in several neurological disorders including Alzheimer's disease, Parkinson's disease, amylotropic lateral sclerosis (ALS) and psychiatric disorders including major depression, and schizophrenia (O'Farrell and Harkin, 2017).

The rate-limiting enzymes in the pathway are indoleamine 2, 3 dioxygenase (IDO) and tryptophan 2, 3 dioxygenase (TDO). IDO is ubiquitous throughout the body and is known to be activated by inflammatory cytokines (Zunszain et al., 2012; O'Connor et al., 2009; Carlin et al., 1989), whereas TDO expression is primarily restricted to the liver with limited expression in the brain, primarily restricted to astrocytes (Miller et al., 2004). Interferon (IFN)γ, a pro-inflammatory cytokine, is a potent inducer of IDO (Pemberton et al., 1997). In the brain the pathway is compartmentalised, within microglia and astrocytes (Guillemin et al., 2001). Both microglia and astrocytes possess IDO which catalyses the conversion of tryptophan to kynurenine, however unlike astrocytes, microglia preferentially express the enzymes, kynurenine monooxygenase (KMO) and kynureninase (KYNU), which lead to the production of the free radical producing metabolites, 3-hydroxykynurenine and 3-hydroxyanthranillic acid respectively and subsequently the production of the NMDA receptor agonist and excitotoxin, quinolinic acid (Guillemin et al., 2003, 2001). As such,

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KP activation in microglia may have consequences for neuronal viability, complexity and function.

Microglial-mediated neurotoxicity is well established and reviewed elsewhere see Block et al. (2007). In the current investigation, we aimed to extend these findings by assessing the impact of KP activation in microglia on neuronal complexity and to elucidate a role for kynurenine-related metabolites in mediating microglial-associated neuronal atrophy. Excess glutamatergic stimulation is a feature of many pathological conditions and manifests in neuronal atrophy and shrinkage as early indicators of eventual neurodegeneration and cell death (Xie et al., 2013; Knight and Verkhratsky, 2010). The use of primary cortical neurons *in vitro* represents a means to investigate the potential toxicity of KP metabolites on neuronal cells. In particular, immature cortical neurons in culture are sensitive to excess glutamate, which is manifested by alterations in neuritic morphology rather than cell death. These glutamate-mediated alterations in neuritic structure may have profound effects on neuronal function, and by extrapolation to central nervous system (CNS) function, influence synaptic transmission (Doucet et al., 2015).

2. Materials and methods

2.1. Reagents

3,4-Dimethoxy-*N*-[4-(3-nitrophenyl)-2-thiazolyl]benzenesulfonamide [Ro 61–8048] and MK-801 were obtained from Tocris Bioscience (UK). Gene expression assays for IDO, KMO, KYNU, and β-actin and Taqman master mix were obtained from Applied Biosystems. Cell culture reagents were obtained from Invitrogen (Ireland), and all other reagents were obtained from Sigma (UK) unless otherwise stated. Anti-βIII-tubulin was obtained from Promega, UK, Alexa Fluor 488 goat anti-mouse was obtained from Invitrogen, USA. Vectashield mounting medium with DAPI was obtained from Vector laboratories (UK).

2.2. Cell culture

The immortalised murine microglial cell line, BV-2 which has similar morphological and functional characteristics when compared with those of primary microglia (Bocchini et al., 1992) was used in these experiments to ensure the purity of microglial cells. At present *in vitro* studies published involve the use of mixed glial cultures of which the proportion of astrocytes to microglia is usually in favour of astrocytes [60–70%; (Hansson, 1984)] with common microglia isolation protocols yielding approximately 10% of microglia from a mixed glial culture (Giulian and Baker, 1986). BV-2 cells were maintained in T75 cm^2 flasks at 37 °C in a 5% CO_2 humidified atmosphere in Roswell Park Memorial Institute medium [cRPMI, 1% (v/v) penicillin-streptomycin, 0.1% (v/v) fungizone, 10% (v/v) foetal bovine serum]. The media was changed every 3–4 days and cells were passaged when 90% confluent. Prior to experiments, cells were plated onto 24-well plates at a density of 1×10^6 cells/ml. Cells were allowed to adapt for 24 h prior to any experimental procedures. All cells used in experiments were between passage numbers 5 and 25.

IFNγ, as a potent inducer of IDO (Yasui et al., 1986), was used as the inflammatory stimulus for these experiments. The concentration of IFNγ was based on previous work undertaken in our laboratory demonstrating that at this concentration, IFNγ has no effect on the viability of either primary cortical neurons (DIV 3) or BV-2 microglia. Mizuno et al. (2008) report that IFNγ (100 ng/ml) directly induces neuronal toxicity in primary neuronal cultures but this dose is considerably higher when compared with the dose of 5 ng/ml used in these experiments.

Specifically, BV-2 microglia-mediated neurotoxicity has been established. Hornik et al. (2016), demonstrated that pheochromocytoma (PC12) cells, a cell line of neuronal origin, were phagocytosed when co-cultured with stimulated BV-2 cells. Additionally, lipopolysaccharide (LPS)-stimulated BV-2 microglia exacerbate oxygen glucose deprivation-induced cell death of mouse organotypic hippocampal slices (Girard et al., 2013). Furthermore, Chen et al. (2011), reported that exposure of the murine motor neuron cell line, NSC-34 to conditioned media (CM) from IFNγ-stimulated BV-2 microglia, increased cytotoxicity after 48 h, as measured by lactate dehydrogenase (LDH) release.

Cultures of primary cortical neuronal cells were prepared as previously described (Day et al., 2014, McNamee et al., 2010) from postnatal day 1 neonate Wistar rat pups (Comparative Medicine Unit, Trinity College, Dublin 2, Ireland) under sterile conditions in a laminar flow hood. Research involving animals in Trinity College Dublin (TCD) is governed by Directive 2010/63/EU on the protection of animals used for scientific purposes in accordance with the requirements of the S.I No 543 of 2012 and reviewed and approved by the Animal Research Ethics Committee prior to submission to the Health Products Regulatory Authority (HPRA) for regulatory approval. Pups were decapitated and the brain was isolated from the skull. The surrounding meninges and obvious blood vessels were removed. Cortical tissue from both hemispheres was isolated from the rest of the brain and was placed in a drop of pre-warmed Neurobasal media [cNBM, 1% (v/v) penicillin-streptomycin, 0.1% (v/v) fungizone, 1% (v/v) glutamax (Gibco) and 1% (v/v) B27 (Gibco)]. The cortical tissue was cross chopped and placed in 5 ml trypsin-EDTA for 2 min. Following this, 5 ml Dulbecco's modified Eagle medium: F12 [cDMEM, 1% (v/v) penicillin-streptomycin, 0.1% (v/v) fungizone, 10% (v/v) foetal bovine serum] was added and the solution was triturated quickly and centrifuged at 3000 g for 3 min at 21 °C. The supernatant was removed and the pellet of cells was resuspended in 5 ml cDMEM and triturated to a homogenous suspension. This suspension was then passed through a cell strainer (40 μm filter) and the suspension was centrifuged at 3000 g for 3 min at 21 °C. The supernatant was removed and the pellet was resuspended in 1 ml cNBM. Cells were incubated in a humidified atmosphere containing 5% CO_2:95% at 37 °C for 3 days before treatment. This protocol yields 97% pure cultures of primary neurons, as demonstrated by Neu-N immunocytochemistry (Minogue et al., 2003).

2.3. Cell culture treatments

Following preparation, stock solutions were filter-sterilised using a 0.2 μm syringe filter. Aside from Ro 61–8048 and quinolinic acid which were dissolved in dimethyl sulfoxide, or recombinant IFNγ which was dissolved in 0.1% bovine serum albumin, all stock solutions were dissolved in cNBM. Quinolinic acid was prepared freshly prior to use to avoid any freeze-thaw effects.

2.4. Reverse transcription polymerase chain reaction (PCR)

RNA was extracted from BV-2 microglia using the Nucleospin® RNA II total RNA isolation kit. Following quantification using a Nanodrop™ 1000 spectrophotometer (Thermo Fisher Scientific, USA), RNA concentrations were equalised and reverse transcribed into cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems). Real-time PCR was performed using an ABI StepOne 7500 instrument as previously described (Frodl et al., 2012). Taqman Gene Expression Assays containing primers and a Taqman probe were used to quantify each gene of interest. Assay ID's for the genes examined are as follows β-actin (4352341E), IDO (Mm00492586_m1), KMO (Mm00505511_m1) and KYNU (Mm00551012_m1). PCR was performed in PCR plates in a 10 μl reaction volume (4 μl of diluted cDNA, 1 μl of Taqman gene expression assay, and 5 μl of Fast Taqman® Universal PCR master mix (Applied Biosystems) and PCR (50 cycles) using ABI universal cycling conditions. Fold change in gene expression from the control group was calculated using the ΔΔCt method, and β-actin served as the endogenous control to normalise gene expression data (Livak and Schmittgen, 2001). The Ct values of β-actin were

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